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(54) Title: PRODUCTION OF NOVEL BOVINE RESPIRATORY SYNCYTIAL VIRUSES FROM cDNAs

(57) Abstract

Disclosed is a synthetic cDNA which codes for an infectious bovine respiratory syncytial virus (BRSV). The cDNA may be derived from a single strain of BRSV, or it may be a chimeric cDNA which is derived from more than one strain of BRSV. The invention also includes vectors and host cells containing the cDNA, methods of producing infectious BRSV using the cDNA, methods of producing attenuated BRSVs and vaccines comprising such attenuated BRSVs.

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Production of Novel Bovine Respiratory Syncytial Viruses from cDNAs

Introduction

Bovine respiratory syncytial virus (BRSV) is a major cause of respiratory tract disease in calves. Respiratory disease is the single most important disease problem of the cattle industry. Seroepizootiologic studies have demonstrated that exposure of cattle to BRSV can vary from mild upper respiratory tract infection to a severe and sometimes fatal interstitial pneumonia.

The genome of BRSV is a single strand of nonsegmented negative-sense RNA of approximately 15,000 nucleotides. Intracellularly, the genomic RNA is transcribed into 10 mRNAs, which encode at least 10 proteins. Of the 10 virus proteins, 8 are structural and 2 are non-structural. Three proteins are contained in the nucleocapsid; specifically the major nucleocapsid protein (N) the phosphoprotein (P), and the large polymerase subunit (L). Three proteins are integral membrane proteins which form the external envelope spikes, namely the attachment glycoprotein (G), the fusion protein (F) and the small hydrophobic protein (SH) of unknown function. There are 2 additional internal virion proteins, namely the matrix (M) protein and a 22 kilodalton protein (M2(ORF1)). The genomic RNA is tightly bound by the major nucleocapsid protein (N) and also is associated with the phosphoprotein (P) and the large polymerase subunit (L). This RNA-protein complex comprises the functional nucleocapsid, which is active in template-dependent RNA synthesis and is packaged in the virion. The M2(ORF1) protein is associated with the nucleocapsid. The functions of the two remaining non-structural proteins (NS1 and NS2) are not clear.

BRSV follows the general scheme of transcription and replication of other nonsegmented negative-strand RNA viruses. The polymerase enters the genome at a promoter in the 3' extragenic leader region and proceeds along the entire length by a sequential stop-start mechanism during which the polymerase remains template bound and is guided by short consensus gene-start (GS) and gene-end (GE) signals. This generates a free leader RNA and 10 nonoverlapping subgenomic mRNAs. The abundance of the various mRNAs decreases with

increasing gene distance from the promoter. The genes are separated by short intergenic regions which are not copied into the individual mRNAs. The 3' terminus (leader) and the 5' terminus (trailer) of the genomic RNA contain the cis-acting sequences important for replication, transcription, and the packaging of viral RNA (vRNA). RNA replication occurs when the polymerase somehow switches to a readthrough mode in which the transcription signals are ignored. This produces a complete encapsidated positive-sense replicative intermediate that serves as the template for progeny genomes. A schematic of the genetic map of BRSV is shown in Fig. 1.

The nucleotide sequences for all the 10 mRNAs and all the intergenic sequences of BRSV strain A51908 (ATCC accession no. VR-794) are known. Samal et al. *Virology* **180**: 453-456 (1991); Samal et al. *J. Gen. Virology* **72**: 1715-1720 (1991); Zamora et al. *J. Gen. Virology* **73**: 737-741 (1992); Zamora et al. *Virus Research* **24**: 115-121 (1992); Mallipeddi et al. *J. Gen. Virology* **73**: 2441-2444 (1992); Pastey et al. *J. Gen Virology* **76**: 193-197 (1995); Mallipeddi et al. *J. Gen. Virology* **74**: 2001-2004 (1993); Yunus et al. *J. Gen. Virology* **79**: 2231-2238 (1998), each of which is hereby incorporated by reference. The 45-nucleotide leader sequence at the 3' end and the 162-nucleotide trailer sequence at the 5' end are also known. However, even with all of the knowledge regarding the genetic make-up and the viral mechanism of action of BRSV, it has not been possible to produce a cDNA of the virus, or generate a stable seed of the virus for the subsequent production of attenuated BRSV for vaccines.

A major reason why it has not been possible to produce a cDNA of BRSV is due to the fact that BRSV is a nonsegmented negative-stranded virus. Unlike DNA and positive-stranded RNA viruses, genetic manipulation of negativ - stranded RNA viruses has been difficult. This is because (i) genetic recombination has not been detected for these viruses and (ii) the naked RNA (i.e., the vRNA) is not infectious. The vRNA is always tightly wrapped by nucleocapsid proteins and is thought to be the minimum unit of infectivity.

Therefore, a recombinant cDNA which insures production of infectious virus in a host cell requires that the cDNA encode not only the vRNA, but also the appropriate viral proteins for transcription and replication to start the first round of virus-specific mRNA synthesis in the host cell.

A few years ago, a breakthrough occurred with the development of methods for introducing individual synthetic genome segments by reassortm nt into influenza virus, a segmented negative-stranded RNA virus. Luytjes et al. *Cell* **59**: 1107-1113 (1989); Enami et al. *Proc. Natl. Acad. Sci. USA* **87**: 3802-3805 (1990), hereby incorporated by reference. However, this breakthrough did not solve the problem at hand, because nonsegmented negative-stranded RNA viruses posed additional difficulties because these viruses do not undergo reassortment. Therefore, these viruses required manipulation of the genome as a single piece.

To overcome these additional obstacles, two alternative approaches were developed for nonsegmented negative-stranded RNA viruses. In one approach, synthetic "minigenomes" consisting of genomic terminal sequences surrounding a reporter gene were transcribed from cDNA *in vitro* and transfected into cells infected with wild type helper virus. Collins et al. *Proc. Natl. Acad. Sci. USA* **88**: 9663-9667 (1991); Park et al. *Proc. Natl. Acad. Sci. USA* **88**: 5537-5541 (1991); Collins et al. *Virology* **195**: 252-256 (1993); Dimock et al. *J. Virol.* **67**: 2772-2778 (1993); De et al. *Virology* **196**: 344-348 (1993), each of which is hereby incorporated by reference.

The second approach involved co-expression of minigenomes and necessary nucleocapsid proteins from transfected plasmids using the transient vaccinia virus/T7 RNA polymerase expression system. Pattnaik et al. *Cell* **69**: 1011-1020 (1992); Calain et al. *Virology* **191**: 62-71 (1992); Calain et al. *J. Virol.* **67**: 4822-4830 (1993); Conzelmann et al. *J. Virol.* **68**: 713-719 (1994); Grosfeld et al. *J. Virol.* **69**: 5677-5686 (1995), each of which is hereby incorporated by reference. These approaches have made it possible to begin the characterization

of cis- and trans-acting factors required for transcription and replication of several nonsegmented negative-stranded RNA viruses.

However, the prior difficulties were not totally overcome by the new methods, since each synthetic vRNA used represented only a fraction of the parental full-length vRNA. The next challenging step was to determine whether these techniques could be used to rescue full-length vRNA analogs. Recently, the second approach was used to recover complete infectious recombinant virus from full length cDNA for several nonsegmented negative-strand RNA viruses, namely, rabies virus (Schnell et al. *EMBO J.* **13**: 4195-4203 (1994)), vesicular stomatitis virus (Lawson et al. *Proc. Natl. Acad. Sci. USA* **92**: 4477-4481 (1995); Whelan et al. *Proc. Natl. Acad. Sci. USA* **92**: 8388-8392 (1995)), human respiratory syncytial virus (HRSV) (Collins et al. *Proc. Natl. Acad. Sci. USA* **92**: 11563-11567 (1995)), measles virus (Radecke et al. *EMBO J.* **14**: 5773-5784(1995)), Sendai virus (Garcin et al. *EMBO J.* **14**: 6087-6094 (1995)), and rinderpest virus (Baron et al. *J. Virol.* **71**: 1265-1271 (1997)), each of which is hereby incorporated by reference.

Production of infectious BRSV from cloned cDNA would be useful to provide a stable vaccine seed. Presently, there is no satisfactory live attenuated or inactivated vaccine available for prevention of BRSV infection. Subunit vaccines have also not been effective against BRSV infections. Live attenuated vaccines are inexpensive and have been effective against many viral infections. However, at the present time, live attenuated BRSV vaccines are made empirically, and the molecular basis of attenuation is not presently known.

Empirically made live attenuated vaccines are currently made via point mutations on isolated virus particles. Vaccines manufactured according to current procedures may be ineffective or even dangerous since the point mutations can revert back after passage in animals, resulting in active virus. Furthermore, since the starting material is different in each case, it is difficult to achieve desirable levels of attenuation; some viruses are over-attenuated and others are under-

attenuated. To make matters more complicated, since there is a high frequency of mutation in RNA viruses, the presently-available attenuated BRSV vaccine stocks are not stable.

An object of the present invention is to produce a cDNA of BRSV which, when inserted into a host cell, is used to produce a stable supply of infectious BRSV. The harvested virus may be used for introducing empirical point mutations into the virus to produce more consistent attenuated BRSV vaccines.

Summary of the Invention

The present invention is therefore directed to a synthetic cDNA which codes for an infectious BRSV. The cDNA may be derived from a single strain of BRSV, or it may be a chimeric cDNA which is derived from more than one strain of BRSV. This aspect of the invention also includes vectors and host cells containing the cDNA.

The invention also includes a method of producing infectious BRSV, comprising inserting a synthetic cDNA which codes for an infectious BRSV into a host cell, and expressing the cDNA in the host cell to produce the infectious BRSV. Optionally, the infectious BRSV may thereafter be purified or isolated. This aspect of the invention also includes an infectious BRSV produced by the method.

The invention further includes a vaccine, comprising an infectious BRSV which has been attenuated by introducing at least one RNA point mutation thereon, wherein the infectious BRSV has been produced by inserting a synthetic cDNA which codes for the infectious BRSV into a host cell, and expressing the cDNA in the host cell to produce the infectious BRSV.

Also included is a method of producing an attenuated BRSV, the method comprising inserting a synthetic cDNA which codes for an infectious BRSV into a host cell, expressing the cDNA in the host cell to produce the infectious BRSV,

and thereafter introducing at least one RNA point mutation into the infectious BRSV to attenuate the BRSV.

The invention also includes the use of an attenuated BRSV in the manufacture of a vaccine against BRSV, wherein the attenuated BRSV is produced by inserting a synthetic cDNA which codes for an infectious BRSV into a host cell, expressing the cDNA in the host cell to produce the infectious BRSV, and thereafter introducing at least one RNA point mutation into the infectious BRSV to attenuate the BRSV.

Brief Description of the Drawings

Fig. 1 is a genetic map of BRSV. Genes are identified according to encoded protein (listed within the boxes). Each gene begins with a 9-nucleotide gene-start signal and terminates with a 12- to 13-nucleotide gene-end signal. Between gene-end and gene-start, there are intergenic sequences. The gene-start of the L gene is located 68 nucleotides upstream of the M2 gene-end.

Fig. 2 is a diagram showing additions and deletions of nucleotides at the 3' end of BRSV vRNA analogs.

Fig. 3 is a diagram showing deletions of 5, 10 and 15 nucleotides in the leader region.

Fig. 4 is a schematic of infection and transfection with plasmid DNAs.

Fig. 5 is a comparison of the leader and trailer regions of BRSV with that of HRSV. The leader and trailer sequences of HRSV are from Mink et al. *Virology* **185**: 615-624 (1991).

Fig. 6 shows the complementarity between the 3' and 5' ends of BRSV genomic RNA.

Fig. 7 shows the structures of RSV vRNA analogs containing a CAT marker gene. Fig. 7A shows the structure of HRSV-CAT(+) cDNA; Fig. 7B shows the structure of BRSV-CAT(+) cDNA; Fig. 7C shows the structure of BRSV-CAT(-) cDNA.

Fig. 8 is a schematic of the RNA transfection and passage experiments disclosed in the present application.

Fig. 9 shows the construction of cDNA encoding BRSV-CAT-F-M2, an analog of BRSV (+)sense RNA containing the CAT, F and M2 genes.

Fig. 10 shows the construction of BRSV antigenomic cDNA and the structure of the encoded proteins.

Fig. 11 shows the cDNA markers in the cDNA-encoded antigenomic RNA.

Fig. 12 is a schematic of the transfection and recovery system for rescuing infectious BRSV from cDNA.

Detailed Description of the Invention

The term "synthetic cDNA" as used herein means that the cDNA is produced by recombinant DNA technology using scientific techniques known in the art. Preferably, the synthetic cDNA in accordance with the invention is produced by independently preparing several cDNA segments of the BRSV genome, and thereafter ligating the BRSV cDNA fragments to form the functional BRSV cDNA which codes for infectious BRSV.

The cDNA may be derived from a single strain of BRSV, or the cDNA may be a chimeric cDNA which is derived from more than one strain of BRSV. Preferably, the cDNA is from one strain, and more preferably the strain is BRSV strain A51908. However, it is well within the scope of the invention to produce a cDNA containing several ligated cDNA fragments from several different BRSV strains.

The cDNA may be inserted into a vector, such as a plasmid. Preferably, pBR 322 is used as the plasmid vector because large size inserts are more stable in this plasmid. The cDNA can also be inserted into a host cell. DH10B cells (Life Technologies) are preferred in this regard because these cells cause minimal rearrangement of large size plasmids.

Another subject of the invention is a method for producing infectious BRSV.

In this method, a synthetic cDNA in accordance with the invention is inserted into a host cell in such a way that the cDNA may be expressed by the host cell (i.e., the cDNA is operably-linked to a promoter), and the cDNA is thereafter expressed in the host cell, whereupon infectious BRSV is produced. The term "operably-linked" broadly includes promoters which are already present in the host cell, or promoters which are physically linked to or inserted independently (at the same or different times) with the cDNA. Useful promoters in this regard are well known to those of skill in the art (Sambrook et al. *Molecular Cloning, A Laboratory Manual*, CSH Laboratory Press (1989); Ausubel et al. *Current Protocols in Molecular Biology*, Greene Publishing and Wiley Interscience, N.Y. (1991), each of which are hereby incorporated by reference). The promoter for T7 RNA polymerase is preferred. The host cell in this regard is preferably a bovine turbinate (Btu) cell. The infectious BRSV produced in accordance with the method may thereafter be purified or isolated from the host cell and/or the culture medium. Also included within the scope of the present invention is infectious BRSV produced in accordance with the method of the invention described above.

Subject matter of the present invention is also a vaccine comprising an infectious BRSV produced in accordance with the invention which has been attenuated by introducing at least one RNA point mutation thereon. Point mutations on virus particles may be made by chemical treatment, heating, drying, irradiation, or any other method in accordance with knowledge of the art.

The invention will be further described by the following Examples.

Example 1: Construction and rescue of BRSV minigenomes

A cDNA was constructed to encode a 953-nucleotide, internally deleted version of BRSV genomic RNA, (BLT(-)), in which the viral genes were replaced with the bacterial chloramphenicol acetyl transferase (CAT) reporter gene. The CAT gene was flanked in turn by sequences representing (i) noncoding

sequences of the first and last genes in the BRSV genome, (ii) BRSV gene-start and gene-end sequences, and (iii) 3' leader and 5' trailer sequences of BRSV genomic RNA. A second cDNA, BLT(+), is a positive-sense RNA which would correspond to the predicted replicative intermediate of BRSV-CAT genomic RNA. The procedure for constructing the cDNA was as follows.

i. Nucleotide sequences of 3' leader and 5' trailer regions of BRSV strain A51908

RNAs with 3 extra Gs, an extra GGGAC, or 11 extra nucleotides and a deletion of 1 or 5 nucleotides at the 3' terminus were tested for promoter activity (Fig. 2). Mutant plasmids were generated by PCR with primers containing these additions and deletions between the T7 promoter sequence and the 3' terminus sequence of BRSV leader region. The presence of these additions or deletions in the plasmids was confirmed by DNA sequencing. Transcription from T7 promoter caused insertion of 3 non-viral G residues at the 3' end of transcripts. This was considered as the wild-type promoter. Therefore, only the addition of an extra 2 and 8 nucleotides between T7 promoter sequence and the 3' end of the leader sequence was necessary. CAT activity and RNA replication of all constructs after transfection and passage was examined. CAT activity below 50% of that of the wild-type promoter was considered significant. RNA replication was analyzed by Northern blot. The RNA bands on the Northern blots were quantitated using a phospho imager. To confirm whether the additions and deletions of nucleotides at the 3' end of the vRNA were retained during replication, the 3' and 5' ends of the RNA template were sequenced following 5'-3' RNA ligation and RT-PCR (*see* Mandl et al. *Biotechniques* **10**: 485-486 (1991)). Briefly, total RNA from one dish of a 6-well plate was purified using Trizol reagent. The total RNA was decapped with tobacco acid pyrophosphatase (Epicentre Technologies). The decapped RNA was circularized by use of RNA ligase (*see* Romaniuk et al. *Methods Enzymol.* **100**: 52-59 (1983)), cDNA

synthesis (*see* Gubler et al. *Gene* **25**: 263-269 (1983)) across the ligated termini, and amplification of the junction region by PCR.

The 3' leader region of BRSV is 45 nucleotides, which contains the promoter sequence for transcription and replication. To define the minimum promoter sequences, plasmids were generated with progressively larger internal sequence deletions. In BRSV and other negative-stranded RNA viruses there is terminal complimentarity, which was retained in all deletion constructs. Three plasmids were constructed in which deletions of 5, 10 and 15 internal nucleotides of the leader region were made (Fig. 3). These constructs were generated by PCR. Effects of these deletions on transcription and replication was examined by CAT assay and Northern blot analysis, respectively.

The 5' trailer region contains the promoter sequence necessary for synthesis of negative sense RNA. To define the minimum promoter sequence, plasmids were generated with progressively larger upstream sequence deletions. Initially, 3 plasmids were constructed in which deletions of 50, 75 and 150 internal nucleotides of the trailer region were made. These constructs were generated by PCR. Effects of these deletions on transcription and were examined by CAT assay.

The BRSV proteins necessary for the various steps in the BRSV growth cycle were identified using a recombinant vaccinia virus T7-based expression system (*see* Fuerst et al. *Mol. Cell. Biol.* **7**: 2538-2544 (1987)). cDNA clones of the 10 BRSV genes were placed in T7-based plasmids, and correct expression has been confirmed for all genes.

In this system, the cDNA was transfected into cells. T7 polymerase was supplied by infection with a vaccinia-T7 recombinant virus and transcription was performed intracellularly. The cDNA was modified to contain a self-cleaving ribozyme motif. Accordingly, the BRSV-CAT constructs were modified to contain a hepatitis delta virus (HDV) genomic ribozyme sequence (*see* Perrotta et al. *Nucl. Acids Res.* **18**: 6821-6827 (1990); Perrotta et al. *Nature (London)* **350**: 434-

436 (1991)) in place of the Hga I site to execute self-cleavage. Addition of the ribozyme sequence was done by 3 successive PCRs. In the first round of PCR, the forward primer contained Hind III and T7 promoter sequences and the reverse primer contained 20 nucleotides of the leader plus 40 nucleotides on the 3' side of the ribozyme cleavage site. In the second and third rounds of PCR, the same forward primer and new reverse primers were used to add the rest of the ribozyme sequence followed by T7 terminator sequence and a Kpn I site. The final PCR product was digested with Hind III and Kpn I and cloned into the Kpn I-Hind III window of pUC 19. The entire sequence was confirmed by DNA sequencing.

It was confirmed that the N, P and L proteins are sufficient for RNA replication. Briefly, Mandin-Darby bovine kidney (MDBK) cells were infected with the recombinant vaccinia virus vTF7-3 (multiplicity of infection of 10) which expresses the T7-RNA polymerase (obtained from ATCC, Herndon, VA). One hour after infection, cells were transfected using LipofectACE, with BRSV-CAT(-) plasmid and various combinations and relative molar amounts of T7-based plasmids containing N, P and L genes. Cells and supernatants were harvested 48 hours after infection for CAT assays and Northern blot analysis using (-) RNA probe (Fig. 4), which hybridizes only to (+) sense RNA made from BRSV-CAT(-) RNA. Conversely, to detect the synthesis of (-) RNA from antigenomes, cells were transfected with BRSV-CAT(+) plasmid and probed with (+) sense probe.

To determine the role of each BRSV protein in transcription and replication, all BRSV proteins were coexpressed in various combinations and relative molar amounts from cDNA using the vaccinia virus/T7 RNA polymerase expression system. Briefly, the cells were infected with vaccinia virus recombinant vTF7-3. One hour after infection, cells were transfected with BRSV-CAT(+) plasmid and various combinations of T7-based plasmids containing BRSV genes. Cells and supernatants were harvested 48 hours after infection for CAT assays and Northern blot analysis.

To determine the BRSV proteins required for formation of virus-like particles, cells were infected with vTF7-3 for 1 hour and cotransfected with BRSV-CAT(+) plasmid and various combinations of T7-based plasmids containing BRSV genes for 3 hours. Then the transfection medium was replaced with growth medium containing 100 µci/ml of ^{3}H-uridine. Forty-eight hours after infection, cells were harvested by scraping and were pelleted by centrifugation at 8000 times g for 1 minute in a microcentrifuge. 100 µl of the supernatants were immunoprecipitated with antisera to N, G and F proteins, and with normal rabbit serum. The immunoprecipitates were collected with Staph. prot. A. and counted in a liquid scintillation counter.

Nucleotide sequences of the 3' leader and 5' trailer regions

The nucleotide sequences of the 3' extragenic leader and 5' extragenic trailer regions were determined for genomic RNA (vRNA) of BRSV strain A51908 (Fig. 5). To sequence the 3' leader region, vRNA was extracted from purified virions and ligated to a synthetic RNA of known sequence. cDNA was made using reverse transcription (RT)-PCR. The 3' leader of BRSV is 45 nucleotides. The 5' trailer region of BRSV vRNA was determined by 5' RACE method. Both G and C trailing reactions were performed to determine the 5' terminal nucleotide. The 5' trailer of BRSV is 162 nucleotides in length. The 3' and 5' ends of BRSV vRNA are partially complementary (Fig. 6), suggesting that similar sequences at the 3' end of the genome and antigenome function similarly during replication.

ii. Construction of BRSV-CAT(+) and BRSV-CAT(-) cDNA

BRSV-CAT(+) cDNA was constructed from RSV-CAT cDNA (available from Dr. P.L. Collins, NIH, Bethesda, MD). A cDNA clone that contained the NS1 gene and leader region of BRSV and was used to obtain BRSV leader sequences was amplified by PCR. The forward primer contained a Kpn I site, T7 promoter and 3' terminal 20 nucleotides of the leader region. The reverse primer contained an

Xba I site and the last 20 nucleotides of the NS1 non-coding region. The amplified product was digested with Kpn I and Xba I and then was used to replace the Kpn I-Xba I fragment of RSV-CAT (Fig. 7A). Similarly, a cDNA clone that contained the downstream portion of the L gene and the trailer region of BRSV was amplified by PCR. The forward primer contained a Pst I site and 20 nucleotides of the L gene non-coding region and the reverse primer contained Hind III and Hga I sites and 5' terminal 20 nucleotides of the trailer region. The amplified product was digested with Hind III and Pst I and was then used to replace the Pst I-Hind III fragment of the previous construct. Conventional conditions of PCR, gel purification, ligation and cloning were followed. *See* Ausubel et al. *Current Protocols in Molecular Biology*, Greene Publishing and Wiley Interscience, N.Y. (1991). The sequence of the BRSV-CAT(+) (Fig. 7B) was confirmed by dideoxynucleotide sequencing.

BRSV-CAT(-) cDNA was constructed from BRSV-CAT(+) by PCR (Fig. 7C). The forward primer contained a Kpn I site, T7 promoter and 5' terminal 20 nucleotides of the trailer region. The reverse primer contained Hind III and Hga I sites and 3' terminal 20 nucleotides of the leader region. The amplified product was digested with Kpn I and Hind III and then cloned into Kpn I-Hind III window of a modified form of pBluescript II KS+ that lacked the T7 promoter. The sequence of BRSV-CAT(-) was confirmed by dideoxynucleotide sequencing.

iii. *In vitro* transcription and transfection

Hga I digested plasmid DNA (2 µg/100 µl) was transcribed *in vitro* with T7 RNA polymerase according to protocols provided by the supplier (Promega). Transcription was for 3 hours at 37° C.

MDBK, bovine turbinate (Btu), and 293 cells were examined for high consistent expression of CAT. All these cell lines support growth of BRSV. A schematic of the RNA transfection and passage experiments are shown in Fig. 8. Briefly, cells maintained in monolayer cultures in 6-well dishes in minimal

essential medium (MEM) containing 10% fetal bovine serum (FBS) was infected with 5 to 10 PFU of BRSV per cell in a volume of 1 ml per well and incubated for 1 hour at 37° C. The cells were washed twice with Opti-MEM 1 (Life Technologies) and incubated for 3 hours at 37° C with a mixture containing 1 ml of Opti-MEM 1, 12 µl of LipofectACE (Life Technologies) and approximately 2-5 µg of *in vitro*-synthesized RNA (2 to 10 µl of transcription reaction). The transcription mixture was removed, the cells were washed once with growth medium (MEM supplemented with 10% FBS and 2 mM glutamine) and fed with 1.5 ml of growth medium. At 20 to 24 hours after infection, cells were harvested for CAT assays and RNA analysis. Cells were harvested by scraping and a 100 µl aliquot of cell suspension was kept aside for CAT activity. The remaining cell suspension was pelleted by centrifugation at 8000 times g for 1 minute at 4° C in a microcentrifuge. The cell pellet was processed for total RNA purification using Trizol reagent (Life Technologies) following manufacturer's instructions with the modification that after isopropanol precipitation, RNAs were purified additionally with phenol+chloroform mixture and precipitated with ethanol. The RNA pellet was dissolved in 50 µl of water and stored at -80° C until Northern blot analysis.

A 100 µl cell suspension kept aside from the transfection experiment was assayed for CAT activity using standard procedures (*see* Ausubel et al., *supra*; Gorman et al. *Mol. Cell. Biol.* **2**: 1044-1051 (1982)). Briefly, cell lysate was prepared by three cycles of freezing and thawing. Lysates were incubated in the presence of ^{14}C chloramphenicol and acetylation was monitored by thin layer chromatography and quantitated by liquid scintillation counting of excised spots.

Approximately 15 µg of total RNA purified using Trizol reagent was examined by Northern blot analysis. RNAs were separated by electrophoresis on small (7 cm long × 10 cm wide) gels of 1.5% agarose in 0.41 M formaldehyde. Gels were blotted onto nitrocellulose membrane using an alkaline transfer buff r (*see* Thomas *Proc. Natl. Acad. Sci. USA* **77**: 5201-5205 (1980)). RNAs were fixed on the nitrocellulose membrane by UV irradiation (Stratagene),

prehybridized for 6 hours at 65° C in 6 × SSC, 0.2 mg/ml sheared denatured salmon sperm DNA, 5 × Denhardt's solution, and 0.1% sodium dodecyl sulfate (SDS), and hybridized for 12 hours in the same solution containing approximately 5×10^6 cpm ^{32}P-RNA per blot. Blots were washed (2 × 15 minutes) at room temperature in 2 × SSC-0.1% SDS and (2 × 15 minutes) at 65° C in 0.1 × SSC-0.1% SDS. Radiolabeled RNA probes were synthesized under conditions of reduced CTP concentration and in the presence of ^{32}P-CTP as described by the supplier (Promega). The reaction was treated with DNase, extracted with phenol+chloroform and passed through a column of Sephadex G-50 to remove unincorporated label.

iv. Incorporation into virus-like particles

To test whether BRSV-CAT vRNA analog was packaged into virions, supernatants from the infected-transfected cells were clarified of cells, and 100 µl of this supernatant was used to infect fresh cells. For antibody inhibition studies, the anti-BRSV serum or anti-bovine PI3 virus serum was added to the inoculum prior to infection and incubated for 30 minutes at 37° C. At 24 hours after infection, cells were harvested for CAT assays and Northern blot analysis. Inhibition of CAT activity and RNA replication by anti-BRSV serum but not by anti-bovine PI3 serum showed that the vRNA analogs were packaged into virus-like particles.

Example 2: Construction and rescue of BRSV midigenomes

BRSV-CAT is only a fraction of the length of the BRSV vRNA. Therefore, "midigenomes" were generated containing several BRSV genes. The F and M2 genes of BRSV were inserted downstream from the CAT, and produced a BRSV-CAT-F-M2 construct of 3860 nucleotides in length.

To construct the midig nome, the dicistronic F-M2 cDNA (clone 162) containing complete F and M2 genes was used (*see* Zamora et al. *J. Gen.*

Virology **73**: 737-741 (1992)). This cDNA was modified by PCR to contain a Pst I site, gene-end and intergenic sequences followed by the gene-start of the F gene and a Pst I site after the gene-end sequence of the M2 gene (Fig. 9). To avoid any possible nucleotide misincorporation during PCR amplification, Pfu DNA polymerase (Stratagene) and 15 amplification cycles were used.

The F-M2 PCR product was digested with Pst I and inserted into the Pst I site of the BRSV-CAT(+) construct. The F and M2 genes in the BRSV-CAT-F-M2 construct were sequenced using F and M2 primers. Positive-sense transcripts were used because large amounts of positive-sense N-, P- and L-specific RNAs were produced from the transfected protein-encoding plasmids, which can hybridize negative-stranded genomic RNA transcripts containing these genes.

Transfection experiments were carried out with cells which had been infected 1 hour previously with recombinant vaccinia virus vTF7-3. BRSV-CAT-F-M2 positive-sense transcript and plasmids containing genes for protein required to make active nucleocapsids were used for transfection. After 48 hours, transfected cells were examined for expression of F and M2 genes by immunofluorescence and immunoprecipitation using antibodies to F and M2 proteins. (*See* Ausubel et al., *supra*.) Northern blot analysis of total RNA with positive-sense F and M2 RNA probes were used to confirm that transcripts of F and M2 genes were generated intracellularly by the BRSV polymerase protein and the input positive-sense CAT-F-M2 transcript was replicated to negative-sense RNA by the T7 polymerase enzyme expressed from the recombinant vaccinia virus.

Example 3: Construction of BRSV cDNA clone.

A cDNA clone encoding the antigenome of BRSV strain A51908 was constructed from cDNA segments which were synthesized by reverse-transcription (RT)-coupled PCR from virion-derived genomic RNA (Fig. 10).

Genomic RNA was extracted from purified BRSV using Trizol reagent according to the manufacturer's instructions (Life Technologies). RT was carried out using superscript RT (Life Technologies) and PCR was carried out using Pfu polymerase (Stratagene). The leader end was constructed to join the promoter for T7 RNA polymerase which included three transcribed G residues for optimal activity. Transcriptions generated three non-viral G residues at the 5' end. To generate a nearly exact 3' end, the trailer end was constructed to join the HDV antigenome ribozyme sequence followed by tandem terminators of T7 transcription (*see* Perrotta et al. (1991) *supra*).

It has been shown that the noncoding intergenic sequences of RSV are not critical for transcription and replication of vRNA (Kuo et al. *J. Virol.* **70**: 6143-6150 (1996)). Therefore, these intergenic regions can be used to assemble functional BRSV cDNA. Four unique restriction site markers (Fig. 11) were introduced into the intergenic region of the antigenomic DNA by incorporating the changes into the oligonucleotide primers used in RT-PCR. RT-PCR fragments were cloned into a modified version of pBR 322 in which the Pst I-EcoRI fragment was replaced with a synthetic polylinker containing unique restriction sites designed to facilitate assembly. Initially, each cDNA fragment was cloned separately and the correct sequence was confirmed by DNA sequencing. Then all the BRSV cDNA fragments were ligated to form the functional BRSV cDNA.

The antigenomic cDNA was completely sequenced to determine the correct sequence. The sequence data showed misincorporation of two nonviral nucleotides, one in the open reading frame of the NS1 gene and the other in the open reading frame of the M2 gene. To correct these misincorporations, cDNA segments carrying these 2 genes were amplified by RT-PCR. Three independent RT-PCRs for each gene were carried out using only 18 cycles of PCR. These segments were completely sequenced. At least one cDNA segment for each gene was found to contain the correct sequence. The segments containing the correct sequence will be used to replace the NS1 and M2 genes in the cDNA.

pBR 322 was used as the plasmid vector because large size inserts are more stable in this low copy number plasmid. DH10B cells (Life Technologies) were used to carry the functional BRSV cDNA clone.

Example 4: Recovery and characterization of infectious BRSV from cDNA.

The bovine turbinate (Btu) cells were chosen for transfection experiments because these cells are highly permissive for BRSV and vaccinia virus. In addition, these cells can be transfected efficiently by the LipofectACE method (Life Technologies). Initially, we will use the transfection condition that was used to rescue infectious HRSV (*see* Collins et al. *Proc. Natl. Acad. Sci. USA* **92**: 11563-11567 (1995)). Briefly, confluent monolayers of Btu cells in six-well dishes are infected with 1 focus-forming unit per cell of recombinant vaccinia virus strain MVA that expresses T7 RNA polymerase (MVA-T7) (available from Dr. B. Moss, NIH, Bethesda, MD). The MVA strain is a host-range mutant that grows permissively in avian cells, whereas in mammalian cells the virus expresses T7 RNA polymerase but there is no production of infectious virus due to a block at a late stage in virion maturation. A mixture of four plasmids containing the BRSV genes N, P, L, and M2 (ORFI) under the control of the T7 promoter (0.4, 0.4, 0.2 and 0.2 µg per well, respectively) and a fifth plasmid (i.e., p(+) BRSV) encoding the functional BRSV antigenome (0.4 µg) is transfected with LipofectACE as recommended by the supplier (Life Technologies). Cells are incubated in a CO_2 incubator at 32° C. Twelve hours later, the medium is replaced with opti-MEM medium (Life Technologies) containing 2% FBS and 40 µg of cytosine arabinoside per ml to inhibit the replication of vaccinia virus. After 3 days, clarified medium supernatants are passaged onto fresh Btu cells and overlaid with methyl cellulose for staining with monospecific antibodies to BRSV F protein by the horseradish peroxidase method (*see* Murphy et al. *Vaccine* **8**: 497-502) or 1% agarose for plaque isolation. Control transfections include cells that received the support plasmids but no p(+)BRSV, and cells that receive p(+)BRSV but no

support plasmids. Different transfection conditions are tested to achieve the highest level of BRSV recovery. Several BRSV-like plaques are picked from plates that had been overlaid with agarose. Each plaque is further purified by two plaque to plaque isolations. Stocks of each plaque isolate are made in Btu cells for characterization. A schematic of the transfection procedure is shown in Fig. 12.

It will then be ascertained that the recovered virus is BRSV. A plaque neutralization test is performed using polyclonal antiserum raised against wild-type BRSV strain A59108. Methylcellulose overlay and neutral red staining is used in the plaque assay. Wild-type BRSV strain A59108 is used as a positive control and wild-type vaccinia virus is used as a negative control. The size of the plaques derived from recovered BRSV is compared with those of the wild-type BRSV strain A51908.

To verify that the three sequence markers inserted into the functional cDNA are present in the recovered BRSV, reverse transcription of genomic RNA purified from wild-type and recombinant BRSV using primers upstream of each restriction site is carried out. The reverse transcription products are amplified by PCR using an additional primer downstream of each restriction site. The presence of the sequence marker in the recombinant virus is verified by digestion of the PCR products with appropriate restriction enzymes. The PCR products representing the recombinant BRSV contains the expected restriction sites while those representing the wild-type BRSV do not contain the restriction sites. To further confirm the sequence markers, the PCR products are cloned and sequenced. This confirms that the recovered BRSV is produced from cDNA clones and is not a laboratory contamination of wild-type BRSV.

The replication behavior of the recovered BRSV is thereafter compared with that of the wild-type BRSV strain A51908. Briefly, Btu cell monolayers in 25 cm^2 culture flasks are infected with 2 PFU of either virus per cell. One flask every 12 hours is transferred to -70° C. The samples are subsequently thawed and

titrated in parallel by plaque assay. Only 2 PFU of BRSV per cell are used because BRSV does not grow to high titer. This study indicates any difference in the replication behavior between the recombinant and wild-type BRSV.

To compare the proteins synthesized by the recombinant BRSV with those of the wild-type BRSV, Btu cells are infected with recombinant BRSV or wild-type BRSV strain A51908 at a multiplicity of infection of 2 PFU. At 20 hours postinfection, the infected cells are labeled with ^{35}S methionine for 2 hours (*see* Mallipeddi et al. *Arch. Virol.* **115**: 23-36 (1990)). The labeled cells are washed with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer. The lysates are clarified by centrifugation and stored at -70° C. An aliquot of each lysate is immunoprecipitated by a BRSV-specific polyclonal antiserum (*see* Mallipeddi et al., *supra*). Total infected cell lysates and immunoprecipitated complexes are analyzed by electrophoresis in 12% polyacrylamide gels. The mobilities and relative amounts of the BRSV proteins are compared between the two viruses.

I claim:

1. A synthetic cDNA which codes for an infectious bovine respiratory syncytial virus (BRSV).

2. The cDNA of claim 1, wherein the cDNA is derived from a single strain of BRSV.

3. The cDNA of claim 1, wherein the cDNA is a chimeric cDNA which is derived from more than one strain of BRSV.

4. A vector, containing the cDNA of claim 1.

5. A host cell, containing the cDNA of claim 1.

6. A method of producing infectious bovine respiratory syncytial virus (BRSV), comprising

inserting a synthetic cDNA which codes for an infectious BRSV into a host cell, wherein the cDNA is operably-linked to a promoter; and

expressing the cDNA in the host cell to produce the infectious BRSV.

7. The method of claim 6, further comprising purifying the infectious BRSV.

8. The method of claim 6, wherein the cDNA is derived from a single strain of BRSV.

9. The method of claim 6, wherein the cDNA is a chimeric cDNA which is derived from more than one strain of BRSV.

10. Use of a synthetic cDNA which codes for an infectious bovine respiratory syncytial virus (BRSV) to produce infectious BRSV in a host cell.

11. An infectious bovine respiratory syncytial virus (BRSV), produced by the following method:

inserting a synthetic cDNA which codes for the infectious BRSV into a host cell, wherein the cDNA is operably-linked to a promoter; and

expressing the cDNA in the host cell to produce the infectious BRSV.

12. The virus of claim 11, wherein the cDNA is derived from a single strain of BRSV.

13. The virus of claim 11, wherein the cDNA is a chimeric cDNA which is derived from more than one strain of BRSV.

14. A vaccine, comprising an infectious bovine respiratory syncytial virus (BRSV) which has been attenuated by introducing at least one RNA point mutation thereon, wherein the infectious BRSV has been produced by the following method:

inserting a synthetic cDNA which codes for the infectious BRSV into a host cell, wherein the cDNA is operably-linked to a promoter; and

expressing the cDNA in the host cell to produce the infectious BRSV.

15. The vaccine of claim 14, wherein the cDNA is derived from a single strain of BRSV.

16. The vaccine of claim 14, wherein the cDNA is a chimeric cDNA which is derived from more than one strain of BRSV.

17. A method of producing an attenuated bovine respiratory syncytial virus (BRSV), the method comprising

inserting a synthetic cDNA which codes for an infectious BRSV into a host cell, wherein the cDNA is operably-linked to a promoter;

expressing the cDNA in the host cell to produce the infectious BRSV; and

thereafter introducing at least one RNA point mutation into the infectious BRSV to attenuate the BRSV.

18. Use of an attenuated bovine respiratory syncytial virus (BRSV) in the manufacture of a vaccine against BRSV, wherein the attenuated BRSV is produced by the following method:

inserting a synthetic cDNA which codes for an infectious BRSV into a host cell, wherein the cDNA is operably-linked to a promoter;

expressing the cDNA in the host cell to produce the infectious BRSV; and

thereafter introducing at least one RNA point mutation into the infectious BRSV to attenuate the BRSV.

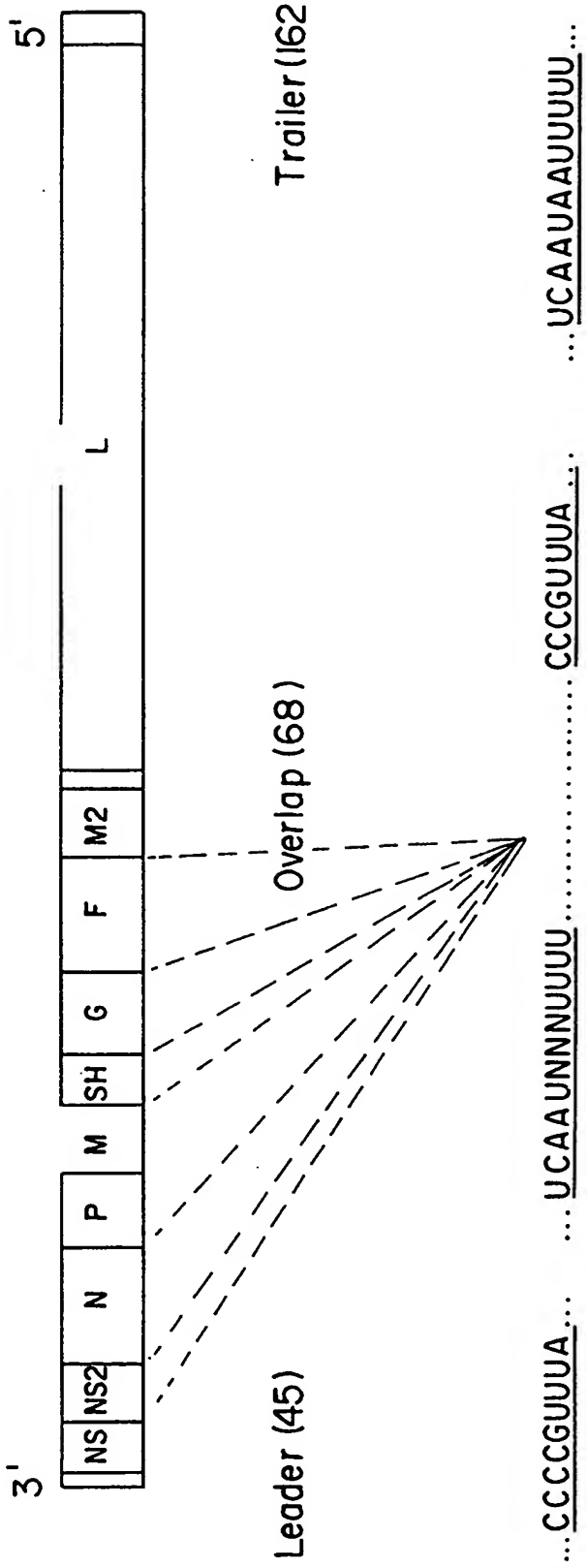


Fig. 1

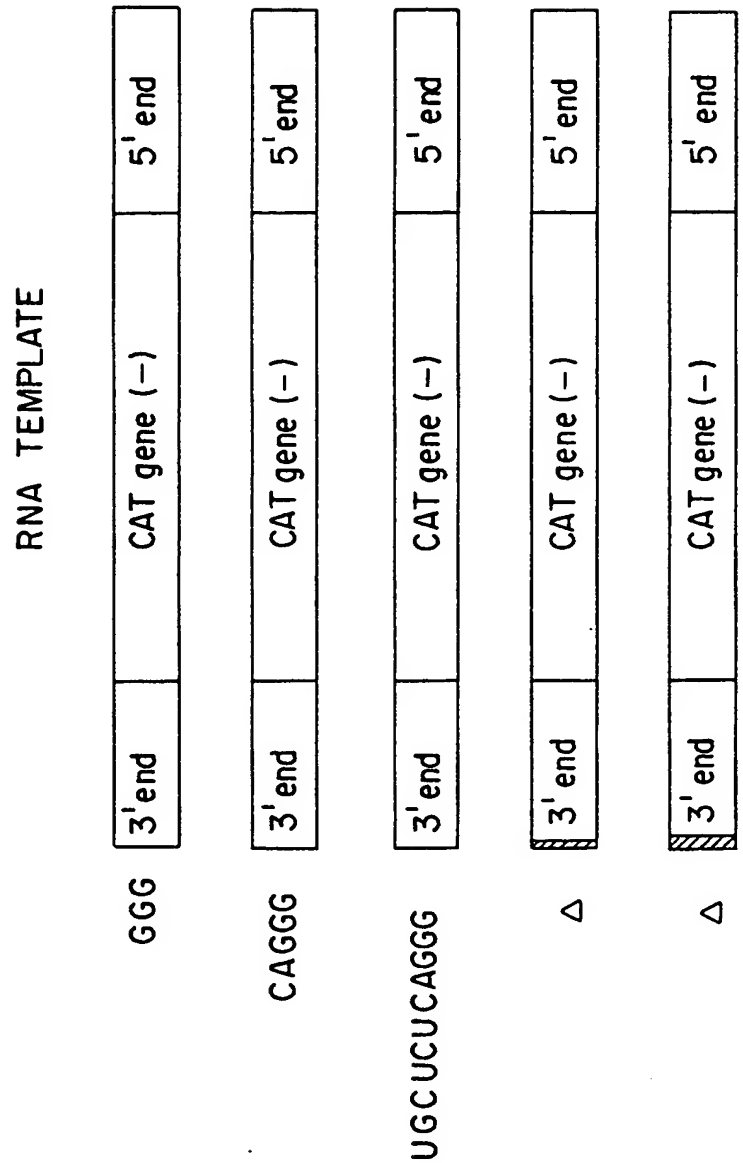


Fig. 2

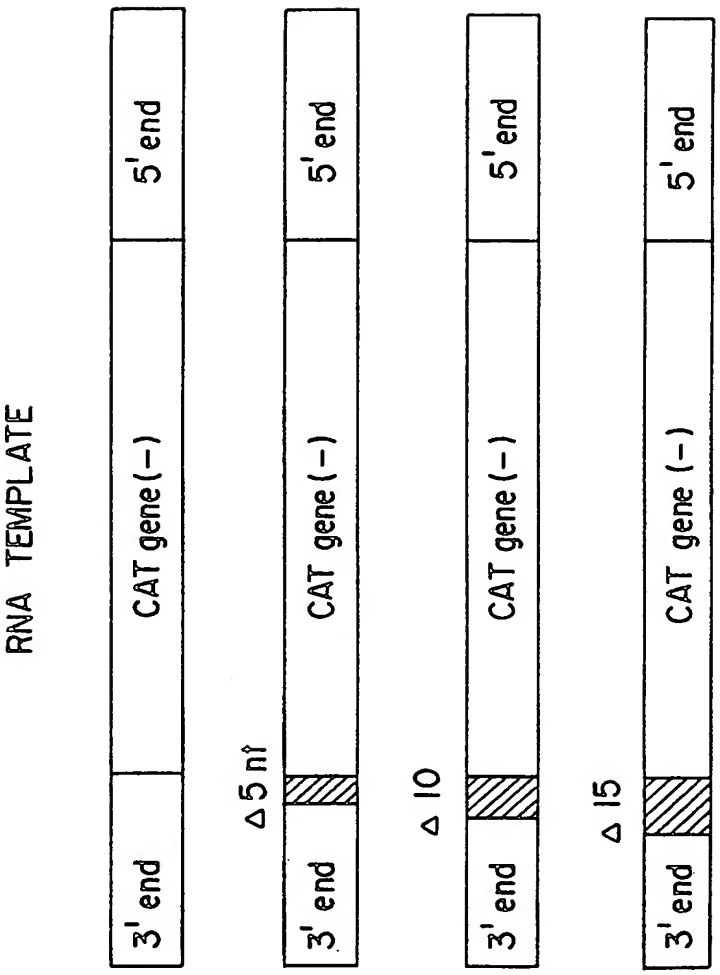


Fig. 3

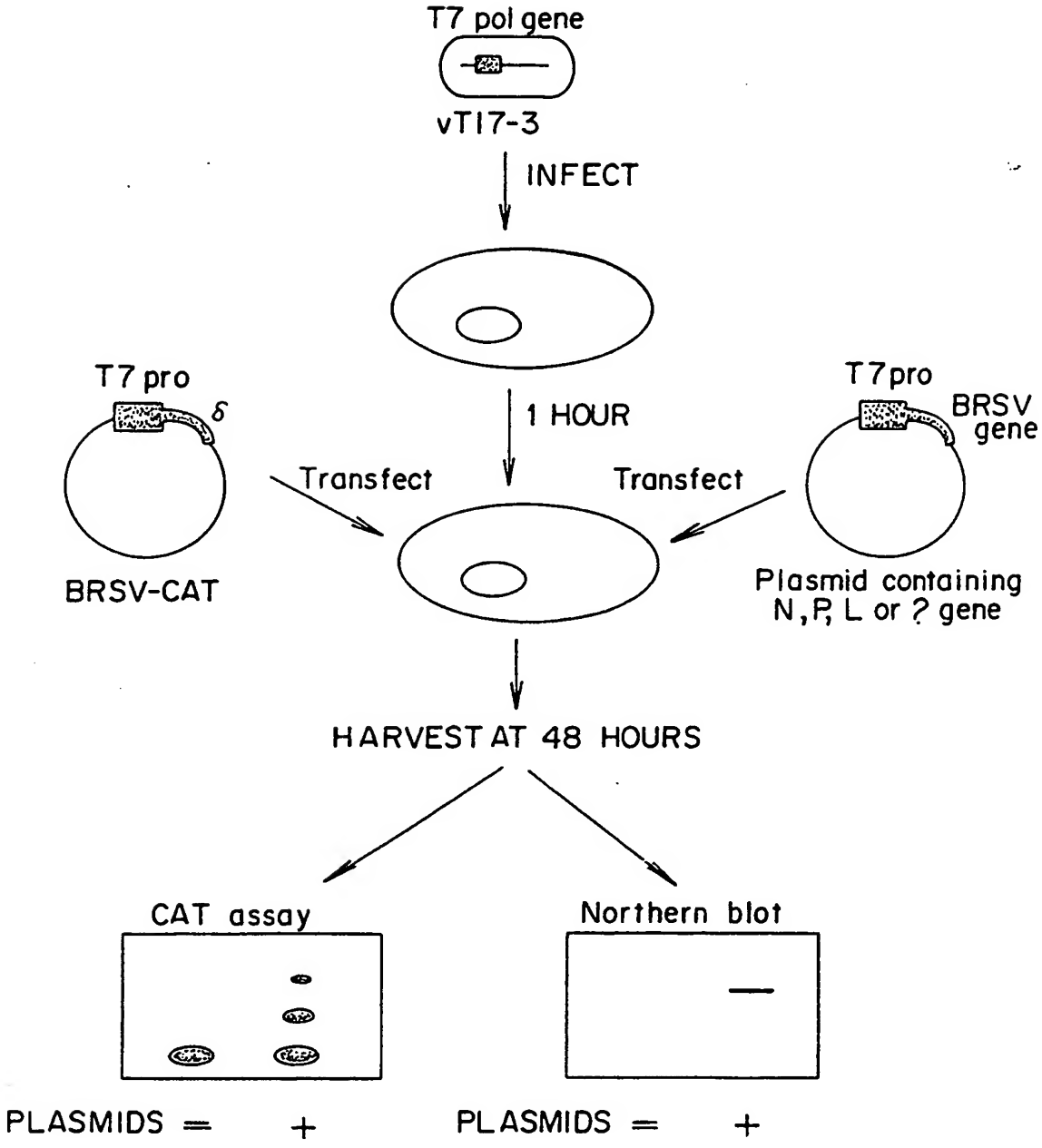


Fig. 4

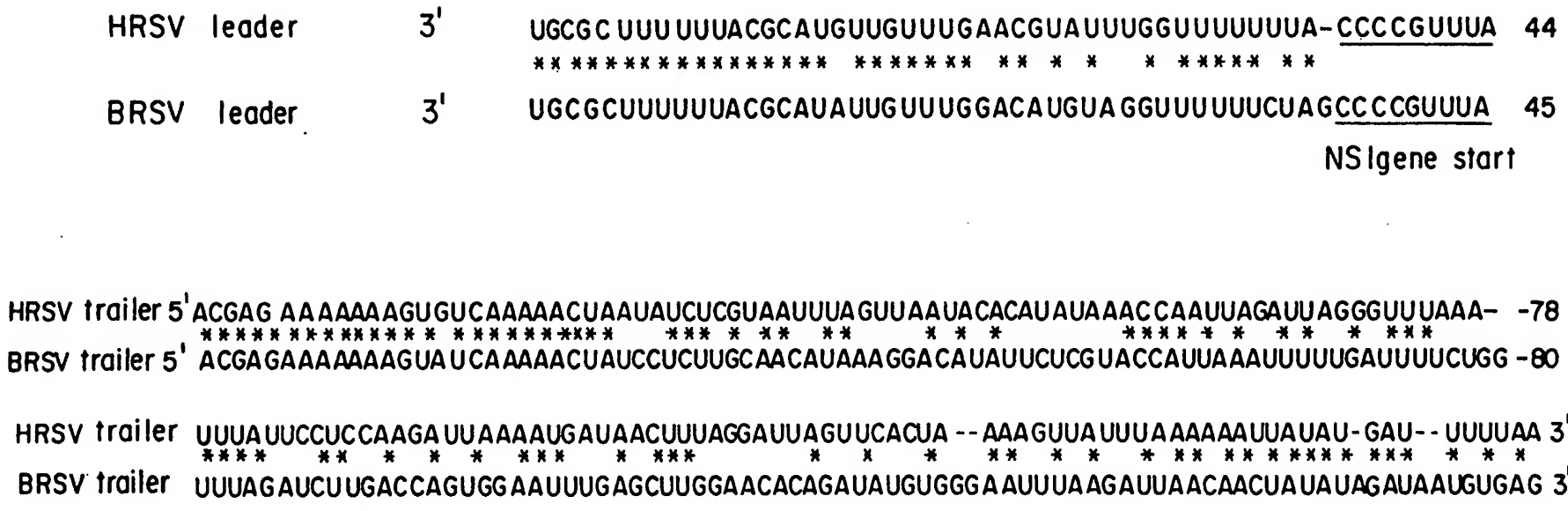


Fig. 5

NS1 gene start

3' UGCGCUUUUUUUACGCAUAUUGUUUGGACAUGUAGGUUUUUUCUAGCCCCGUUUA . . . . . .

*** ******** * * ** **** * *** * *

5' ACGAGAAAAAAAGUAU-CAA-AAACUAUCCUCUUGCAACAUAAAGGACAUAUUC . . . . . .

Fig.6

Fig. 7A

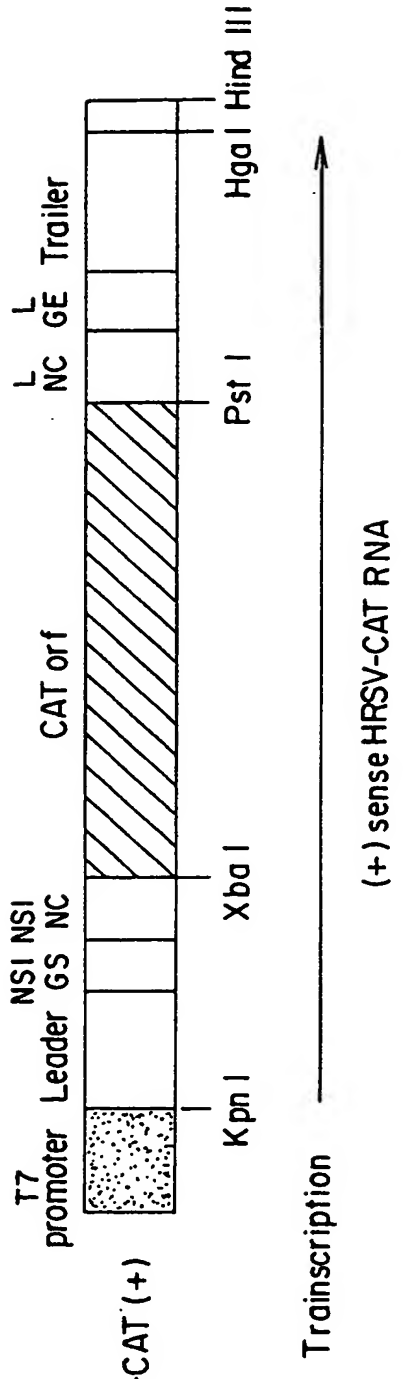


Fig. 7B

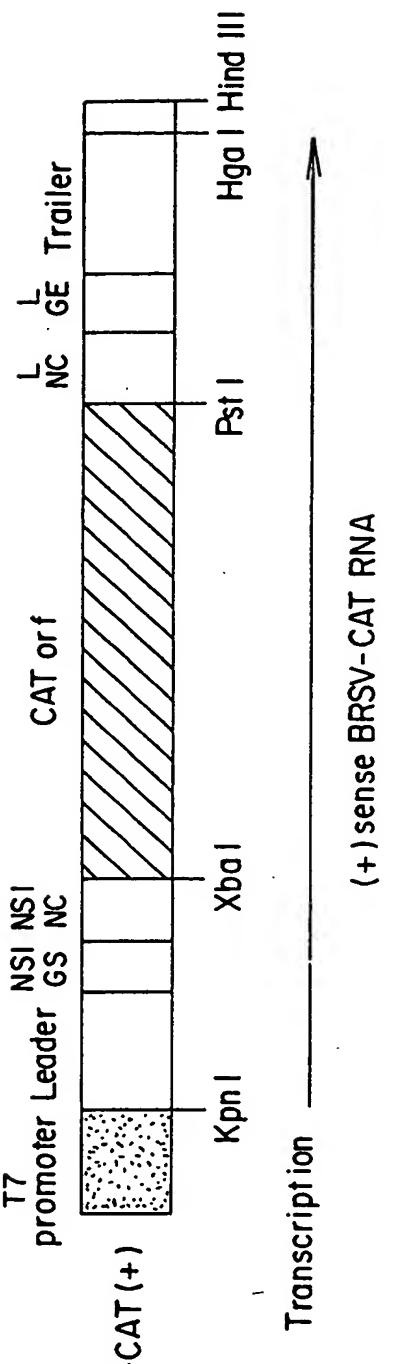
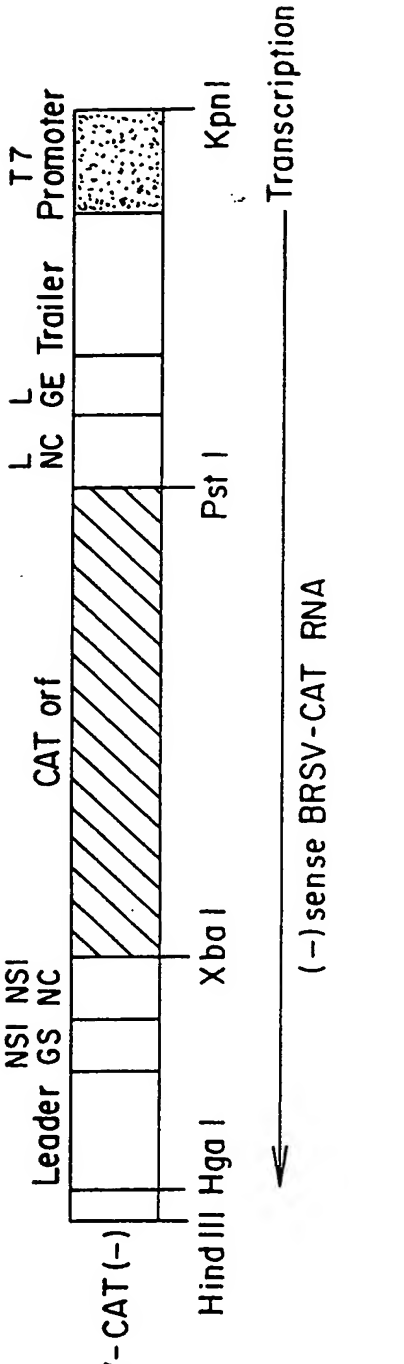


Fig. 7C



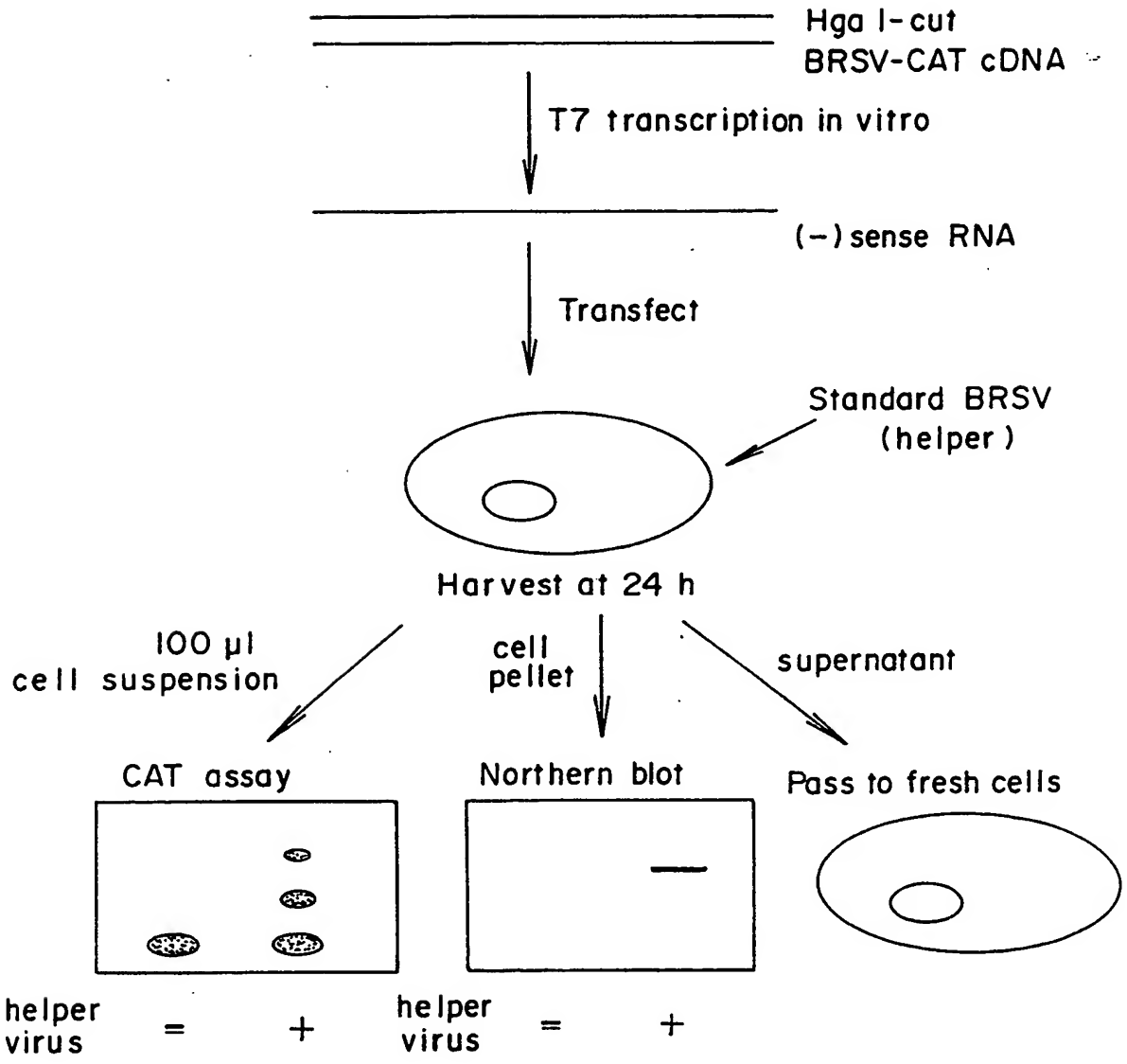


Fig. 8

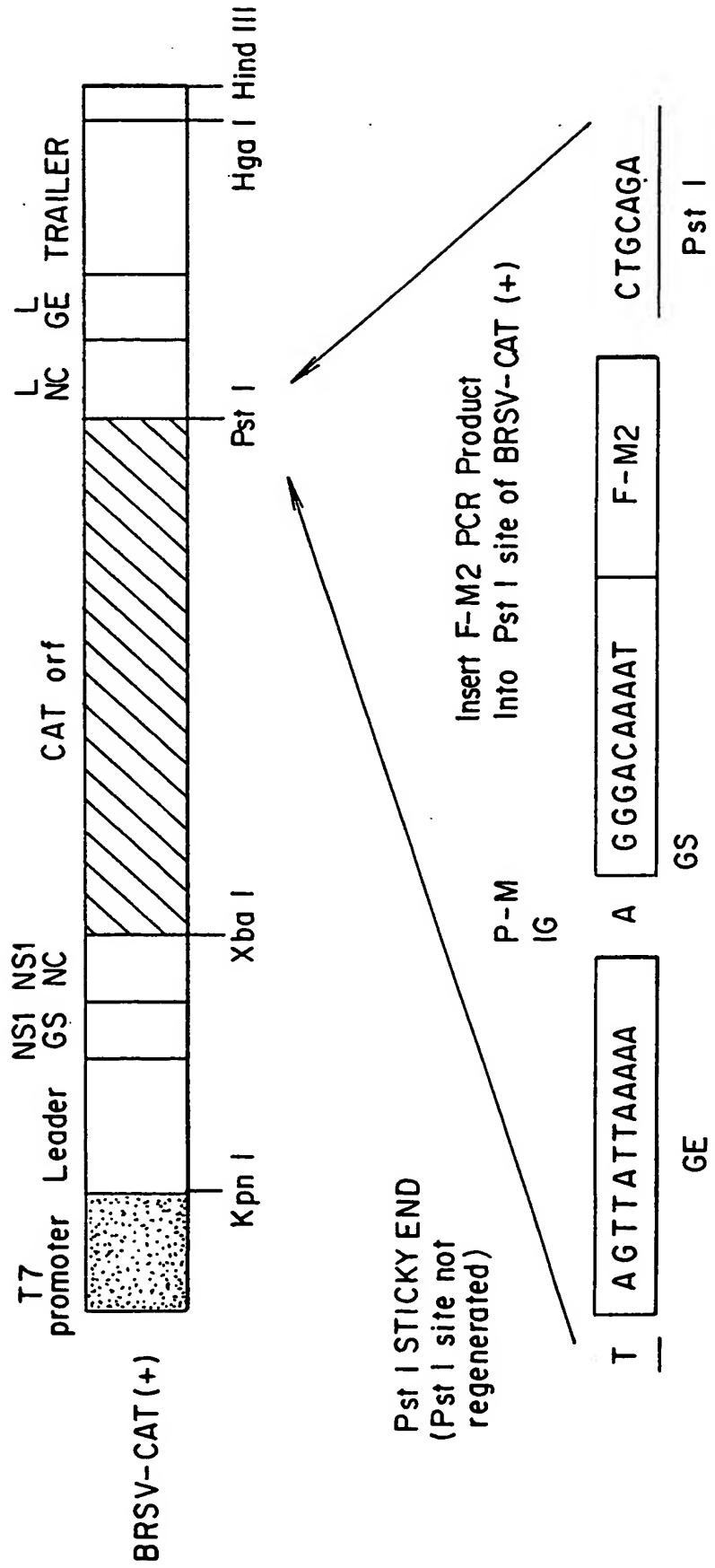
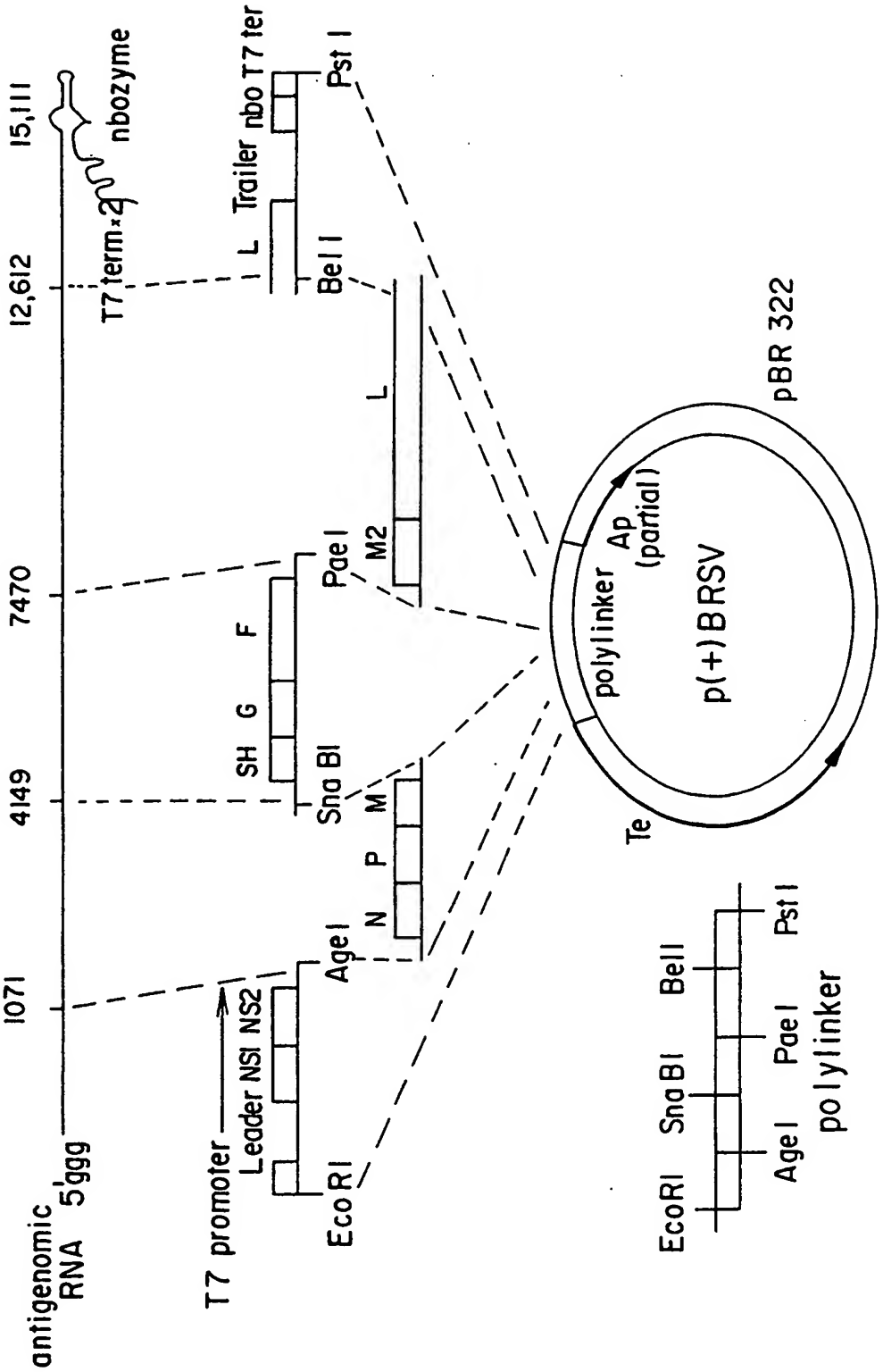


Fig. 9

Fig. 10



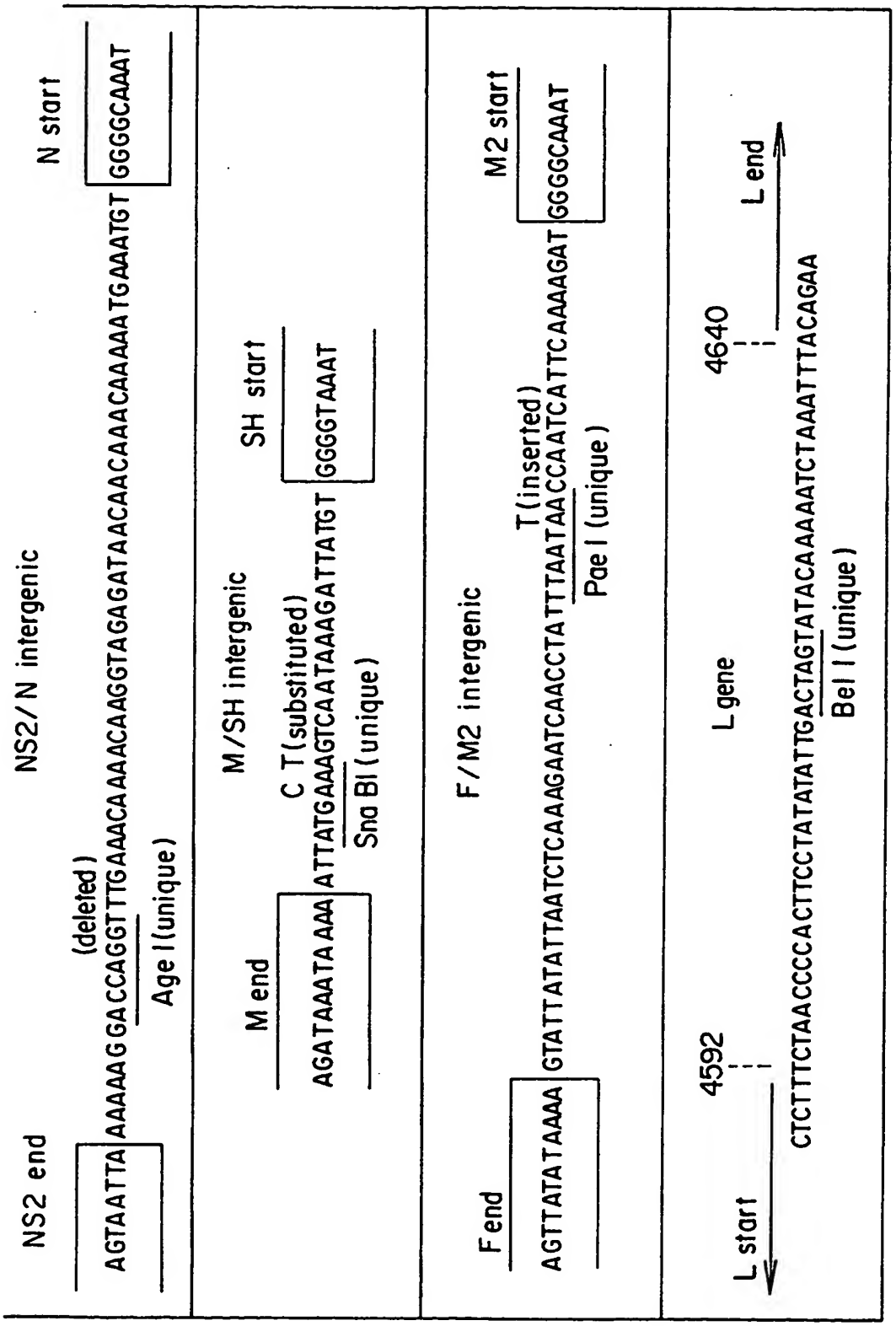


Fig. 11

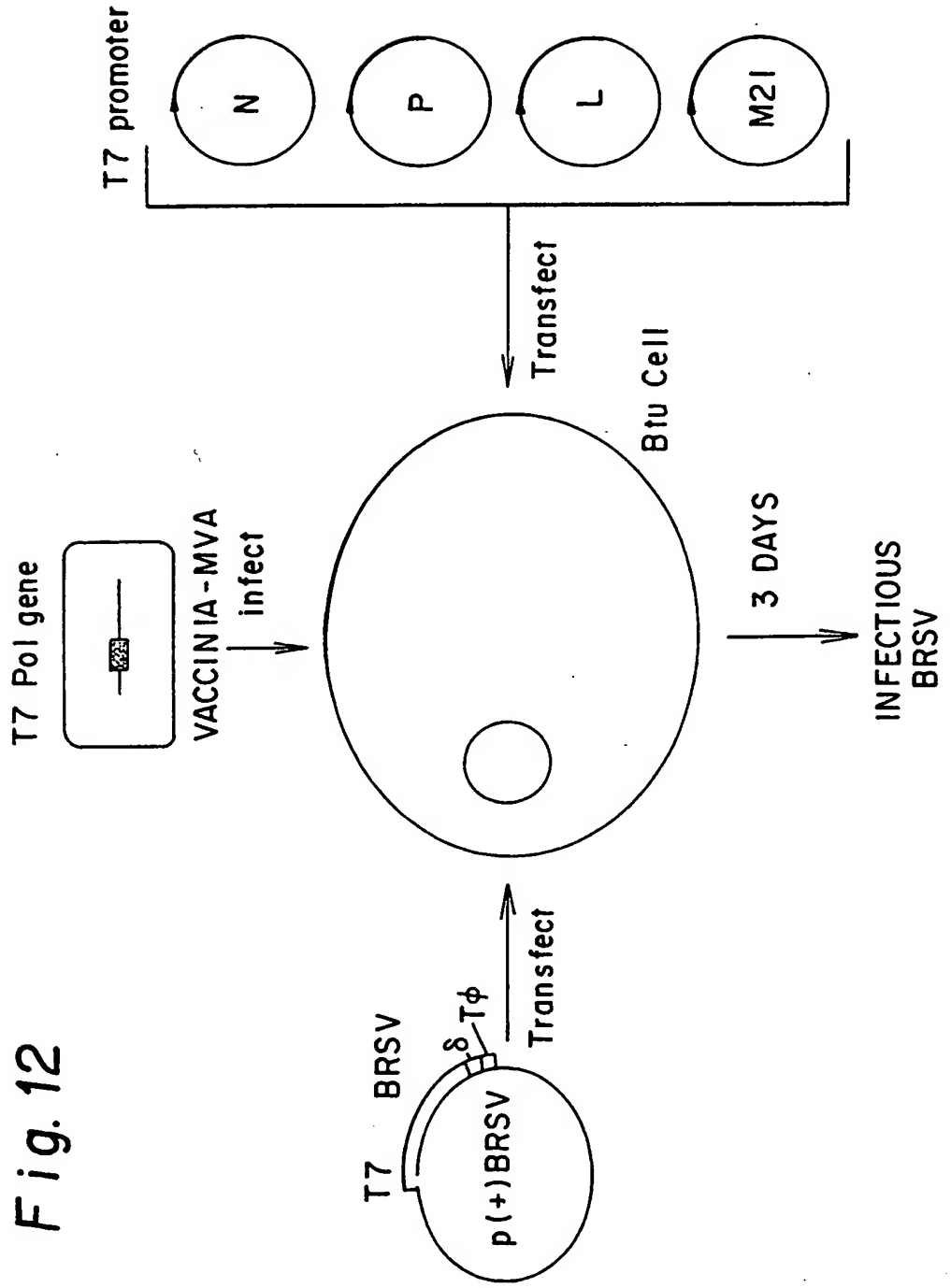


Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23231

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/11, 1/21, 15/85, 15/63, 7/00; A61K 35/00
US CL :536/23.1; 435/240.2, 252.3, 320.1, 235.1; 424 93.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/240.2, 252.3, 320.1, 235.1; 424 93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, WPIDS, MEDLINE, CAPLUS
BRSV, bovine respiratory syncytial virus, DNA, cDNA, vaccine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92/07940 A2 (SAMAL) 14 May 1992, page 5 lines 15-23, page 9, pages 11-15.	1-18
Y	WO 92/01471 A1 (THE UAB RESEARCH FOUNDATION) 06 February 1992, page 1 lines 1-15, page 9 lines 5-32, pages 18-21.	1-18
X, P	US 5,716,821 A (WERTZ et al) 10 February 1998, col 3 line 1 to col 4 line 25 and claims.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
22 MARCH 1999

Date of mailing of the international search report
12 APR 1999

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